DECOLORISATION OF METHYLENE BLUE USING *LACTUCA* AND *SOPHORA* SPECIES

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Dedicated to the loving memory of my father, Anil Dhaneshwar

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I would like to start by thanking **Lord Almighty** for giving me strength and intelligence to pursue my project.

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Abstract

The world is facing numerous environmental problems, and water pollution is being amongst the major ones. Water quality has worsened dramatically due to various pollution sources. Textile industry effluents, containing a variety of natural and synthetic dyes, is one of the chief sources of pollutants. The diverse structures of these dyes lead to many challenges for their degradation. Certain plants have inbuilt potential to remediate these dyes. The aim of this study is to assess the potential of a non-native New Zealand plant (*Lactuca sativa* L. var. Great Lakes) and New Zealand native plants (for example, Kowhai) for phytoremediation of methylene blue (MB) as the model dye. Spectrophotometric analysis showed that 10 seeds of *Lactuca* spp. and pre-germinated *Sophora* spp. were able to decolorize 40 mg/L MB up to 78% in first 48 hours of incubation under non-sterile as well as aseptic conditions. *Lactuca* spp. showed up to 86% decolorisation within 96 hours of incubation. The decrease in the radicle lengths by about 20% indicated toxicity of MB towards plants. Increasing the number of seeds from 10 to 20 resulted in 88% decolorisation within first 48 hours of seed incubation. The decolorisation potential was inversely proportional to the increase in MB concentration with 81% decolorisation of 40 mg/L in 48 hours, whereas 400 mg/L showed only 28% decolorisation in the same time period. Under repeated stress conditions the seeds still had a capacity to decolorise MB but the efficiency decreased from 80% in the first cycle to about 43% in third cycle. Pre-germinating the seeds had a positive effect on their decolorisation capacity at higher MB concentrations, while the presence or absence of seed coat and controlled pH did not significantly affect the decolorisation capacity of lettuce seedlings. Using UV-Vis spectroscopy and cyclic voltammetry, the total absence of MB from the system was confirmed, suggesting degradation of the dye. Significant activity induction of marker bio-transformation enzymes from lettuce radicles indicated their probable role in the metabolism of MB. Phytotoxicity assays using ryegrass seeds and seedlings demonstrated the non-toxic nature of the metabolites following decolorisation of MB by lettuce seedlings, suggesting that lettuce seedlings had dye detoxification capacity.

Introduction and Literature Review

1. General introduction

The world is facing a number of environmental problems of both "green" and "brown" nature (McGranahan & Satterthwaite, 2000). The "green" issues mainly include environmental problems of irrigated agriculture, rain-fed agriculture, forests and rangelands. The "brown" environmental problems stem from industrial wastewater pollution, domestic waste water pollution, motor vehicle emissions, urban and industrial air pollution, etc. (Gadallah & Sayed, 2014). Water pollution is one of the major "brown" problems. Water quality is thought to have worsened dramatically because of pollution from industrial, municipal and agricultural sources (Gadallah & Sayed, 2014; Lee, 2010).

1.1 Pollutants from the textile industry

Textile industry is one of the largest and growing sectors throughout the world (Chang & Lin, 2001), and thus there is a significant increase in the use of synthetic complex organic dyes as the coloring material. The annual world production of textile is about 30 million tons requiring about 100,000 tons of different dyes (Zollinger, 1987). Due to a large quantity of water used in the dyeing process these textile industries are also one of the greatest producers of liquid effluents which are discharged after minimal pre-treatment into the environment with a high amount of pollutants (Oxspring et al., 1996). The industrial effluents containing dyes impede light penetration and are aesthetically displeasing. It may also contain trace metal elements (Banatet al., 1996) which pollute the water when released and cause eutrophication leading to algal boom, death of aquatic life and oxygen deficiency. Release of dyes presents an ecotoxic hazard and introduces the potential danger of bioaccumulation (Saratale et al., 2009; Saratale et al., 2011). They have a variety of toxic effects on the flora and fauna (Raskin et al., 1994; Banat et al., 1996; Nigam et al., 1996; Platzek et al., 1999; Pinheiro et al., 2004; Saratale et al., 2009; Saratale et al., 2011). Thus there is need for a remediation method to tackle this growing problem.

1.2 Effluent treatment methods

The methods for dye removal from wastewater can be sectioned into 3 different categories: physical, chemical and biological. Physical methods include adsorption by activated charcoal,

ionizing radiation, reverse osmosis, microfiltration and ultra-filtration (Lokesh & Sivakiran, 2014), whereas, the chemical methods include techniques like chlorination, ozonation, oxidation & reduction, coagulation, flocculation and floatation (Joshi et al., 2004). These methods are expensive and commercially unattractive. They could also generate wastes (secondary pollution) (Ghodake et al., 2009). These secondary pollutants then require additional treatments for disposal (Miao, 2005). Biological methods are considered to be more cost effective in comparison to physical and chemical methods.

2. Background

2.1 Textile industry and effluent

Textile industries are found in most of the countries with their numbers increasing drastically over the years. Synthetic dyes are being used in the textile, paper, cosmetic, and pharmaceutical and food industries. Because of their ease of use, cost effectiveness in synthesis, as well as stability and variety in color; they are preferred over the natural dyes (Chang & Lin, 2001). The textile industries consume a large amount of water and variety of chemicals for wet processing of textiles. The chemical reagents used are very diverse in composition, ranging from inorganic compounds to polymers and organic compounds (Banat et al., 1996). As a large amount of water is used and there is very little pre-treatment before discharge, the large quantity of waste water generated has a large amount of pollutants (Oxspring et al., 1996). The effluent generated in the textile industry is colored and has high pH and temperature, high chemical oxygen demand (COD), high total dissolved solids (TDS), high total suspended solids (TSS), and may at times highly alkaline (Lokesh et al., 2014). These effluents enter the water body and could either inhibit biological activity or cause a variety of water-borne diseases. The pollutants, aggravated by the presence of free chlorine and toxic heavy metals, cause rapid depletion of dissolved oxygen and also tend to destroy the micro-organisms necessary for maintaining a healthy state of the water body.

2.2 Synthetic dyes

A dye is a colored substance that has an affinity to the substrate to which it is being applied. Dyes contain a chromophore, (delocalized electron system with conjugated double bonds), and an auxochrome (electron withdrawing or electron donating substituent) that cause or intensify the

color of the chromophore by altering the overall energy of the electron system. Most dyes also contain metals which pollute the water when released and cause eutrophication and other consequences (Banat et al., 1996).

Textile dyes are of various types depending on their structures and mode of application (Minussi et al., 2007). They can be classified as (1) acid dyes (e.g. acid red 128), (2) direct (substantive) dyes (direct dye 81), (3) azoic dyes (methyl orange), (4) disperse dyes (disperse orange 1), (5) sulfur dyes (sulfur black 11), (6) fiber reactive dyes (dicholorotriazine), (7) basic dyes (basic red 9), (8) oxidation dyes (hair dyes), (9) mordant (chrome) dyes (mordant blue 3), (10) developed dyes (primuline red), (11) vat dyes (indigo), (12) pigments (Egyptian blue), (13) optical/fluorescent brighteners (blankophor b), and (14) solvent dyes (solvent red 24).

Azo compounds constitute the largest and the most diverse group of synthetic dyes and are widely used in a number of industries such as textile, food, cosmetic and paper printing (Pandey et al., 2007). Azo dyes are the most widely used among synthetic dyes, representing almost 70% of the textile dyestuffs produced (Knackmuss, 1996; Kudlich et al., 1996). They are synthetic colors that contain an azo group, -N=N-, as part of the structure. They are easy to synthesize, of low cost, stable, can be used to color several materials (textile, leather, plastic, food), and allow a great variety of colors and shades. All the azo dyes containing a nitro dye were found to be mutagenic (Chung & Cerniglia, 1992). There exists a clear relationship between the chemical structure and their potential danger.

The biologically active dyes are mainly those compounds containing p-phenylenediamine and benzidine moieties. It was found that methylation of the phenylenediamine component or substitution of a nitro group with an amino group did not decrease mutagenicity of dyes. However, sulphonation, carboxylation or deamination led to a decrease in the mutagenicity (Chung & Cerniglia, 1992; Chung et al., 1992). Stringent government legislations are forcing textile industries to treat their waste effluents to an increasing high standard

2.3 Toxic effects of textile dyes

Synthetic dyes such as azo, xanthenes and anthraquinone dyes are very toxic to living organisms. Azo dyes are enzymatically metabolized by azoreductase to many carcinogenic aromatic amines (Nakayama et al., 1983; Platzek et al., 1999). Aromatic amines could induce urinary bladder

cancer in humans and tumors as well, thus posing a great threat as a carcinogen or a mutagen (Combes & Haveland-Smith, 1982). Furthermore, their discharge into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into water bodies and thereby affecting aquatic life (Pinheiro et al., 2004). Dyes decrease light absorption and may significantly affect photosynthetic activity of aquatic plant life. They are also toxic due to the presence of aromatics and heavy metals (Banat et al., 1996). Dyes being one of the major sources of heavy metals and thus at times they can be phototoxic (Raskin et al., 1994) and can be persistent in nature causing imbalance in the ecosystem.

Numerous azo dyes are used in industries daily of which more than 500 contain potentially carcinogenic aromatic amines in their chemical formulation. They are generally recalcitrant to biodegradation due to their xenobiotic nature. The microorganisms being highly versatile are expected to develop enzyme systems for the decolorisation and mineralization of azo dyes under certain environmental conditions (Pandey et al., 2007). The acute toxicity of azo dyes, as defined by the EU criteria for the classification of dangerous substances, is rather low. Only a few azo dyes showed LD50 (lethal dose that kills half of the tested population) values below 250 mg / kg body weight (Zille, 2005). However, occupational sensitisation to azo dyes has been seen in the textile industry since 1930 (Trizio et al., 1988). Especially some disperse dyes with monoazo or anthraquinone structures have been found to cause allergic reactions, i.e. eczema or contact dermatitis (Hatch & Maibach, 1995).

Following oral exposure, azo dyes are metabolised to aromatic amines by intestinal microflora or liver azoreductases. The soluble aminosulphonates are usually quickly excreted, whereas those derived from aniline, toluene, benzidine and naphtalene have been shown to have carcinogenic properties. However these properties are attributed to further metabolism (oxidation to N-hydroxy-compounds) by mammalian cytochrome P-450 enzymes. For instance, methemoglobinemia results from the oxidation of iron (II) to iron (III) in haemoglobin, which prevents oxygen binding (Lin & Wu, 1974).

2.4 Waste water treatment

Color is among the first contaminants which are noticed by naked eyes. Very small amount of dyes are capable of imparting color to large quantities of water (Patil et al., 2009). Wastewater containing dyes is very difficult to treat, since the dyes are recalcitrant, resistant to aerobic

digestion and are stable in light (Gurulakshmi et al., 2008). Treatment of dye wastewater involves physical/chemical methods like chlorination, bleaching, floatation, coagulation, precipitation (Saratale et al., 2008), adsorption by activated charcoal, ozone oxidation, ionizing radiation, ultrafiltration, etc. (Saratale et al., 2009). The available conventional wastewater treatment systems are unable to completely remove the recalcitrant dyes and other organic residues from textile effluents (Shaul et al., 1991; McMullan et al., 2001). Much research has been focused on chemical and physical degradation of azo dyes in wastewaters (Edwards, 2000). Many of these technologies are cost prohibitive or not very established. And might lead to generation of wastes (secondary pollution), which are of limited application and efficiency and are poorly disposable (Miao, 2005).

2.4.1 Wastewater treatment using biological means

Of all the technologies investigated in waste water treatment, bioremediation has emerged as the most desirable approach for cleaning up of the pollutants. Bioremediation is a pollution mitigation technology that uses biological systems to catalyze the degradation of or transformation of various toxic chemicals to less harmful forms. Biological processes provide an alternative to existing technologies because they are more cost effective, environmental friendly and do not produce large quantities of sludge. It is known that several microorganisms (Saratale et al., 2011) can decolorise and even completely mineralize many azo dyes under certain environmental conditions (Rai et al., 2007).

Fungal systems appear to be useful in the treatment of colored and metallic effluent (Ezeronye & Okerentugba, 1999). *Phanerochaete chrysosporium* has been shown to mineralize a variety of recalcitrant aromatic pollutants. Lignin Peroxidase was found to be responsible for the decolorisation of the azo, triphenyl methane, heterocyclic and polymeric dyes (Pasti-Grigsby et al., 1992; Ollikka et al., 1993). *P. chrysosporium* provided effective decolorisation of wastewater, about 45% decolorisation after only 1 day of treatment, and reaching about 90% decolorisation after 7 days; whilst *Pleurotus ostreatus* was capable to decolorise and detoxify acid wastewater providing a 40% decolorisation after only 1 day. *Trametes hirsute* (Abadullah et al., 2000; Domínguez, 2005), *Phanerochaete chrysosporium* & *pleurotus sajor-caju* (Kumaran and Dharani, 2011) and *Aspergillus flavus* (Lalitha et al., 2011), etc. have also shown dye decolorisation potential. *A. niger* group degraded (RR) Reactive red (93%) and (RG) Reactive

green (80%) under optimum conditions. Studies have also stated that *Kluyeromyces marxianus* IMB3, a yeast strain, is able to decolorise remazol black B (98%) (McMullan et al., 2001).

Bacteria have been increasingly used and considered one of the most preferred choices in bioremediation processes. VT-II, an aerobic gram positive bacillus (*Bacillus spp*.) was found to have maximum observable Congo red (azo dye) decolorisation activity. Under optimal conditions of pH (7.0) and temperature (40° C), maximum decolorisation percentage was found to be 85% (Sawhney & Kumar, 2011). *Citrobacter spp* was isolated from soil treated with effluent and its extracellular culture filtrate showed broad spectrum decolorisation efficiency for azo and triphenylmethane dyes. Zhou and Zimmerman (1993) used actinomyces as an adsorbent for decolorisation of effluents containing anthroquinone, phalocyanine and azo dyes. *Bacillus subtilis* showed 90% decolorisation of Acid blue 113 within 50 h. Maximum dye decolorising efficiency was observed at 200 mg/l concentration of the dye (Gurulakshmi et al., 2008). Three strains of genus *Halomonas* were able to decolorise azo dyes in a wide range of NaCl concentration (up to 20% w/v), temperature (25-40° C), and pH (5-11) after 4 days of incubation in static culture (Asad et al., 2007). The symbiotic nitrogen fixing soil bacterium, Rhizobium species are recognized as economic, easily available choices for degradation of dye compounds. *Rhizobium radiobacter* MTCC 8161 decolorised (90%) a deep red sulfonated diazo dye Reactive Red 141 (50 mg/L). This bacterium decolorised the increasing concentrations of dyes (100-500 mg/l) with a decolorising efficiency varying from 60-90% (Telke et al., 2008). *Sinorhizobium meliloti* possesses the capability to degrade toxic nitroaromatic compounds, such as 2, 4-dinitrotoluene (DNT) (Dutta et al., 2003).

Decolorisation of diazo dye Direct brown MR (DBMR) by *Acinetobacter calcoaceticus* NCIM 2890 was observed effectively (91.3%) in a static anoxic condition, even though a lower growth rate of the bacterium was observed. In comparison, agitated cultures of the bacterium grew well but showed less decolorisation (59.3%) within 48 h of incubation (Ghodake et al., 2009). The *Pseudomonas spp*. decolorised the Direct Black 38 within 5 days under anaerobic condition and *Stenotrophomonas acidaminiphila* (BN-3) decolorised the C.I Acid Red 88 within 60 h (Khehra et al., 2005). Decolorisation of Brown 3 REL by 86% was reported by Waghmode et al. (2012) using *Brevibacillus laterosporus.* While 100% decolorisation of Reactive green 19A was reported by Saratale et al. (2009) using *Micrococcus glutamicus. Aeromonas hydrophila* was

reported to have successfully degraded a number of dyes such as Reactive Red 198, Reactive Black 5, Reactive Red 141, Reactive Blue 171, Reactive Yellow 84 (300 mg L^{-1}) by Hsueh et al. (2009). Green macroalga *Enteromorpha* sp was reported to decolorise C.I. Basic Red 46 by Khataee et al., (2013).

Different approaches to bioremediation take advantage of the metabolic processes of different organisms for degradation. For example, soil bioremediation might be performed under either aerobic or anaerobic conditions, and involve optimization of the metabolic pathways of bacteria or fungi for degradation of hydrocarbons, aromatic compounds or chlorinated pesticides (Haritash & Kaushik, 2009). Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process. Many compounds that are legally considered to be hazardous can be transformed to harmless products (Vidali, 2001). Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible. Bioremediation technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site while ex situ involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are

- Bioventing: a process involving encouraging microorganisms already existing at a contaminated site, to degrade the pollutants, by providing air or oxygen.
- Land farming: A process where contaminated soils, sediments, or sludge in the upper soil zone are incorporated into the soil surface and intermittently turned over to aerate the mixture.
- Bioaugmentation: A process in which strain(s) of microorganism is added to the contaminated site to aid the degradation of the pollutants.
- Rhizofilteration: The process of using plant roots to remove pollutants from contaminated water.
- Biostimulation: A process where the limiting nutrient (like oxygen, carbon etc.) is added to modify the environment to aid the in-situ microorganisms in contaminant degradation.

Phytormemdiation is the use of plants to clean up potentially damaging spills. The plants work with soil organisms to transform contaminants such as heavy metals and toxic organic compounds into harmless forms (Gavrilescu, 2004). The knowledge that plants can also be used to clean up contaminated soil has opened up new avenues for research (Chaudhry et al., 2005). It refers to the natural ability of certain plants called hyper acclumulators, to bio-accumulate the

contaminants (Baker & Boorks, 1989; Seraj et al., 2014). These plants may possess the ability to degrade or render harmful contaminants (Brooks, 1998).

Different plant species can have different enzymatic constitutions and the degradation capacity depends mainly on the presence and induction of the responsible enzyme. Laccase and peroxidases are majorly being explored for their dye decolorisation capacity (Kamath et al., 2004). Laccases are copper containing metalloenzymes found in plants, and microorganisms (Ferrario et al., 2015). They catalyse the oxidation of lignin related phenolic or non-phenolic compunds (Yang et al., 2014). *T versicolor* releases laccase as its major extracellular enzyme, and this is its major mechanism in decolorising anthraquinone, azo and indigo dyes (Wong & Yu, 1999). Laccase was induced in *Brassica juncea*, playing a role in degradation of Reactive red 2 (Ghodake et al., 2009). Lignin peroxidase was found to be responsible for the decolorisation of azo, heterocyclic and polymeric dyes (Pasti-Grigsby et al., 1992). Peroxidases of *Phragmites australis* have been seen to have a role in degradation of Acid orange 7 using (Carias et al., 2008). Significant induction in enzyme activities of laccase (112%) and lignin peroxidase (278%) was reported by Saratale et al. (2009)

2.5 Plants chosen for this study

Lactuca is a genus of plants from *Asteraceae* family mainly growing near the water bodies with a variety of uses (Garg et al., 2004). These plants have the potency to phytoremediate heavy metal-contaminated soils (Gunduz et al., 2012). Owing to its rapid growth, lettuce seed is capable of germinating in less than 12 hours under suitable conditions, making it an ideal model plant for germination experiments. Due to the ability to germinate in a range of light and temperature conditions, experiments could be conducted with relative ease without the need for specialised growth rooms. Due to the relatively high number of treatments required, quick and reliable germination of seedlings was highly important. And thus lettuce was chosen as the model plant for most of the experiments here.

Sophora is a genus of plants from the *Fabaceae* family. Certain species of *Sophora*, commonly known as Kowhai are a native New Zealand species (Song, 2005). Using a native species saves the need and process of introducing any foreign species into the environment. Also being a native species, the plant is already acclimatised to the environmental conditions of the place.

Hence Kowhai was selected an example of New Zealand native plant to investigate the potential use of native plants for dye remediation.

2.6 The dye used

Methylene blue (MB) also known as basic blue 9 is a cationic basic thiazine dye extensively used in textile industries (Gul et al., 2013). Although not strongly hazardous, it is known to cause some harmful effects like vomiting, jaundice, cyanosis and quadriplegia and tissue necrosis on acute exposure (Bulut & Aydin, 2006). Some studies have been done using various means to decolorise MB solutions. Manghabati & Pazuki (2014) reported 90-95% dye removal using Duckweed and *Spirodela polyrrhiza* under favourable conditions (low concentration of MB, high plant weight etc.). Sun & Xu (1997) used sunflower stalks to adsorb MB, with 80% dye removal within 30 minutes. Polyaniline zirconium (IV) silicophosphate nanocomposite was used for sorption removal of MB from water system with a maximum adsorption capacity of 12 mg/g (Gupta et al., 2014). *Oscillatoria* sp. Dominated cyanobacterial mat was used for MB adsorption resulting in maximum adsorption of 78.43 mg/g (Kumar et al., 2012). El Sikaily (2007) reported the potential of *Ulva lactuca* as absorbent for removal of MB under various conditions. Studies using wheat shells and hazelnut shells showed up to 90% dye removal (Bulut & Aydin, 2006; Doğan et al., 2009). There is a paucity of studies on the potential of plants for MB phytoremediation.

2.7 Objectives of this thesis

A wide variety of microorganisms like bacteria (Marimuthu et al., 2013; Omar et al., 2013) and fungi (Swamy & Ramsay 1999; Abadulla et al., 2000; Nyanhongo et al., 2002) have been shown to have remarkable potentials of decolorising and degrading textile dyes. But this method needs the induction of foreign strains of microorganisms into the water body. Thus plants pose as a better option for decolorising and degrading the textile dyes. They increase the visual value of the environment while carrying on phytoremediation. Many plants have been tested for their potential to degrade textile dyes (Patil et al., 2009; Kagalkar et al., 2010; Khandare et al., 2011; Kabra et al., 2012; Khataee et al., 2012; Patil et al., 2012; Jayanthy et al., 2013).

The area of dye degradation using both *Lactuca* and *Sophora* species is yet to be reported. This study aims to assess the potential of *Lactuca* and *Sophora* seedlings to decolorise and degrade

the textile dye Basic blue 9 (MB) under sterile and aseptic conditions and to determine some of the physiological changes and biochemical occurring in the plant body after exposure to the dye as a stressor. This may prove to be an environmentally friendly method of dye degradation without producing any harmful by-products, and in turn may help increase the visual aesthetics of remediation vicinity.

Materials and methods

Experiments were performed to evaluate the amount of decolorisation by lettuce seed germination and kowhai seed germination in the presence of methylene blue (MB).

1. Plant material

Lettuce seeds (*Lactuca sativa* L. variety 'Great Lakes') were purchased from Kings Seeds (Katikati, New Zealand) and were stored within their original aluminum packaging in a fridge kept at 4°C.

Figure 1: Lettuce seeds (*Lactuca sativa* L. variety 'Great Lakes')

Kowhai seeds were collected from several trees grown at a site of the University of Canterbury campus, and stored at room temperature in a plastic container.

Figure 2: Kowhai seeds

2. Dye

Methylene blue used in this study was obtained from Sigma (St. Louis, Missouri, USA).

- Synonym: 3,7-bis(Dimethylamino)phenazathionium chloride, Tetramethylthionine chloride
- Empirical Formula (Hill Notation): $C_{16}H_{18}ClN_3S \cdot xH_2O$

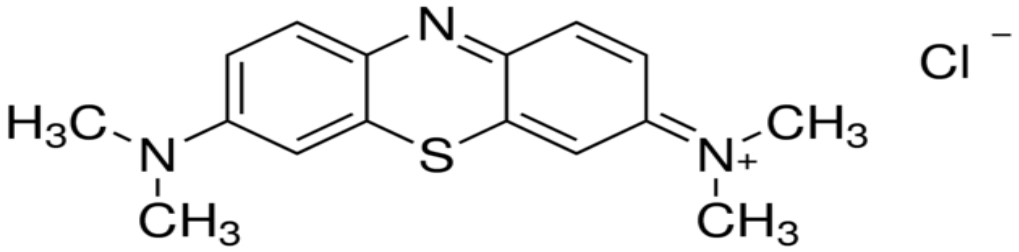

Figure 3: Molecular structure of methylene Blue (adapted from Sigma Aldrich.com)

- Molecular Weight: 319.85 (anhydrous basis)
- CAS Number: 122965-43-9
- Maximum absorption: 670nm

A stock solution of 400 mg/L was prepared (filter sterilised using a Millipore filter) and stored at room temperature.

3. General decolorisation procedure using lettuce seeds

All decolorisation experiments were performed in triplicates and were performed twice. The seeds were left to germinate and grow for 48 hours in a growth room at 25°C, before the percentage of seeds germinated was recorded and the germinated seedlings were removed for further inspection and comparison. Aliquots of 0.5 ml were withdrawn after decolorisation and made up to 1 ml and then absorbance was measured at 670 nm. Decolorisation was expressed in terms of % and was calculated using the following formula:

% decolorisation = [(initial absorbance – final absorbance) / initial absorbance] *100

The data recorded for each parameter measured was used to determine a mean value and standard deviation.

3.1 Decolorisation experiment: A preliminary study

The purpose of this initial experiment was to investigate the general response of lettuce seedlings to MB stress. Five ml of 40 mg/L MB solution, or de-ionised water (control), were placed into a clear 35 ml plastic tube. Ten seeds were then sown on the surface of the solution in each tube.

3.2 Direct treatment experiments

3.2.1 Screening experiment

In a 35 ml clear plastic tube, ten lettuce, ryegrass or wheat seeds were placed on the surface 5 ml 40 mg/L MB dissolved in de-ionised water as control and left to germinate and grow for 48-96 hours in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.2.2 Effect of increasing dye concentrations

Ten seeds were sown on the surface of 5 ml of varying concentrations (40, 80, 120, 160, 200, 240, 280, 320, 360, 400 mg/L) of MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 h in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.2.3 Increased number of lettuce seeds

Twenty seeds were sown on the surface of 5 ml of 40 mg/L MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 h in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.2.4 Effect of repetitive stress exposure

Ten lettuce seeds were sown on the surface of 5 ml 40 mg/L MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 hours in a growth room at 25°C. Seedlings were then removed and re-suspended in 5 ml of fresh 40 mg/L MB solution. This cycle was repeated twice and % decolorisation was recorded at the end of each cycle.

3.2.5 Decolorisation under aseptic conditions

Lettuce seeds were surface sterelised by suspending them in 30% (v/v) a household bleach solution containing 4.2% (w/v) active sodium hypochlorite and then rinsed thoroughly with autoclaved de-ionised water in a laminar air flow hood. Ten lettuce seeds from these were then grown in the presence of 5 ml 40 mg/L MB solution (made up in autoclaved de-ionised water) or autoclaved de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 hours in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.3. Pre-treatment experiments

3.3.1 Effect of pre-germination on increasing dye concentration

Lettuce seeds were germinated in de-ionised water for 48 hours. These seeds were then sown on the surface of 5 ml of varying concentrations (40, 80, 120, 160, 200, 240, 280, 320, 360, 400 mg/L) of MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 h in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.3.2 Effect of seed coat

Lettuce seeds were germinated in de-ionised water for 48 hours and were divided into two batches. Half of them retained the seed coat and the other half had the seed coats removed. These seeds were then sown on the surface of 5 ml of 40 mg/L of MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 h in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.3.3 Effect of spent treatment water

Lettuce seeds were germinated in de-ionised water for 48 hours. The spent water was then used to prepare 40 mg/L MB solution. MB solution prepared in fresh de-ionised water was used as

control. Ten lettuce seeds were germinated on the surface of 5 ml of the this MB solution in a 35 ml clear plastic tube and left to germinate and grow for 48 hours in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.3.4 Increased number of lettuce seeds

Lettuce seeds were germinated in de-ionised water for 48 hours and the seed coat was removed. Twenty seeds were sown on the surface of 5 ml of 40 mg/L MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 h in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

3.4 Treatment under controlled pH conditions

A 0.1 M solution of MES (2-(*N*-morpholino)ethanesulfonic acid) was made up in de-ionised water and pH was adjusted to 6.0 using 0.1 M NaOH. This buffer was then used in place of de-ionised water to repeat the direct treatment and pre-treatment experiments previously described to determine growth effects of MB and its decolorisation at a known pH. .

4. Experiment using Kowhai seeds

Kowhai seeds were surface-sterilised by suspending them in 30% bleach solution and then rinsed thoroughly with autoclaved de-ionised water in a laminar air flow hood. The seeds were then nicked slightly and placed in plastic Petri plates on a sheet of moist filter paper for 48 hours. Then the seeds were germinated in 5 ml 40 mg/L MB solution (made in autoclaved de-ionised water or autoclaved de-ionised water as control in plastic Petri plates and left to germinate and grow for 48 hours in a growth room at 25°C. Seedlings were then removed and radicle length was measured. The percentage of seeds germinated and % decolorisation were also recorded in this experiment.

5. Growth measurements

Due to the relatively small size of lettuce seedlings, measuring the length of seedling parts using a ruler was seemingly impractical. Instead, the length of seedling roots (radicles) was determined

with the help of high resolution photos taken at the end of the experiments, from which radicle length was calculated using ImageJ software (version 1.49q).

After growth in a test solution, seedlings were arranged in a row and photographed on a piece of paper, as shown in Figure 4 below. This method allows for the accurate measurement of growth differences of a few millimeters among lettuce seedlings during early post-germinative growth.

Figure 4: Seedlings after 48 hours MB stress.

6. Enzyme extraction and assays

Ten lettuce seeds were germinated in 5 ml 40 mg/L MB solution or de-ionised water as control in a 35 ml clear plastic tube and left to germinate and grow for 48 hours in a growth room at 25°C. The radicles were excised, weighed and suspended in 50 mM Na-phosphate buffer (pH 7.4). The chopped roots were then ground in a mortar and pestle followed by centrifugation at 8000 rpm for 20 minutes at 4^0C. The cell free extract obtained was used as the intracellular enzyme source. The solution obtained after harvesting the plantlets was used as the extracellular enzyme source (Kagalkar et al., 2009; Khandare et al., 2013) The specific activities of laccase and lignin peroxidase were determined spectrophotometrically for both test and control samples. All the assays were run in triplicates and average rates were calculated.

6.1 Laccase assay

Laccase activity was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS, Sigma Aldrich, St Louis, USA) (molar extinction coefficient 3.6×10^4 $M^{-1}cm^{-1}$) as substrate in a 1.5 ml reaction mixture containing 0.95 ml 0.1 M citrate-phosphate buffer (pH 3.4), 0.05 ml

enzyme extract (see 6) and 0.5 ml 1 mM ABTS. The oxidation of ABTS was detected by measuring the increase in absorbance at 420 nm (Lu et al., 2007). The enzyme control contained 0.05 ml buffer instead of enzyme.

6.2 Lignin peroxidase assay

Lignin peroxidase activity was determined by following the oxidation of 2 mM veratryl alcohol (Sigma Aldrich, St Louis, USA) to veratraldehyde (molar extinction coefficient $9.3\times10^3\ M^{-1}cm^{-1}$) in 0.1 M sodium tartrate buffer (pH 3.0) in the presence of 0.4 mM H_2O_2. The increase in absorbance was monitored at 310 nm (Lu et al., 2007).

7. Protein estimation

The amount of protein in the lettuce enzyme extracts (see 6) was estimated by Folin-Lowry's method (Lowry et al., 1951). A standard curve was prepared by dispensing varying volumes of standard protein solution (bovine albumin, Life Sciences Technology, New Zealand) into labeled test tubes and the volume of each tube was made up to 1 ml using de-ionised water. Five ml of the reagent A (48 ml of 2% Na_2CO_3 in NaOH, 1 ml of 1% NaK tartrate in H_2O and 1 ml of 0.5% $CuSO_4.5H_2O$ in H_2O) was added, thoroughly mixed and was left to stand for 10 minutes. This was followed by the addition of 0.5 ml reagent B (1 part Folin-Phenol: 1part water). The resulting solution was then left to stand for 30 minutes after which absorbance was measured at 660 nm. Similar procedure was carried out for 1 ml of appropriately diluted lettuce enzyme extracts.

8. Cyclic voltammetry

Cyclic voltammograms were recorded on a potentiostat (ADInstruments ML160 Potentiostat). The data recorder unit is an ADInstruments PowerLab 2/20 ML820. The software is ADInstruments EChem v2.5.4. Three electrodes were used; the reference, the auxiliary and the working electrode (figure 5) in a reversible system. The resulting solution obtained after full decolorisation of 40 mg/L MB using 35 lettuce seeds was used to obtain the voltammograms.

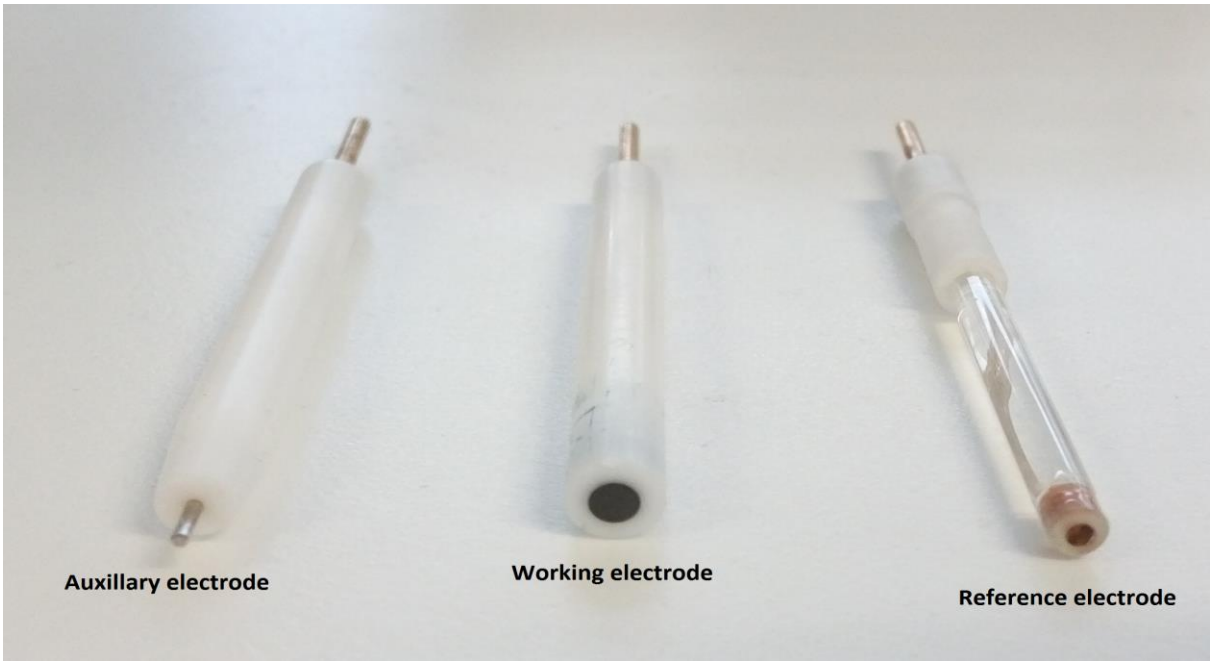

Figure 5: Electrodes used for cyclic voltammetry

9. UV-Visible Spectroscopy

Spectrum of MB (40mg/L) and the resulting solution obtained after full decolorisation of 40 mg/L MB using 30 lettuce seeds was recorded using a UV visible spectrophotometer (Ultraspec 2100 pro UV/Visible spectrophotometer, by Amersham Biosciences), the scan ranged from 200-800 nm.

10. Phytotoxicity studies

Short rotation ryegrass seeds (*Lolium hybridium* Hausska, variety "Moata") obtained from local seed supplier were used for phytotoxicity studies of MB solution after decolorisation treatment with lettuce seedlings.

Figure 6: Ryegrass seeds

The experiment was carried out by placing ten seeds in 5 ml of each fresh MB (40 mg/L), fully decolorised MB solution, spent de-ionised water (used as control in initial decolorisation experiment) and fresh de-ionised water. The radicle length and percent germination were recorded after 72 hours.

11. Data analysis

All analyses were carried out in the statistical package StatPlus (version 5.7.8). In the experiments on the effect of increasing MB concentrations, the results with a linear relationship were analysed using a simple linear regression in R, with significance calculated from a 95% confidence interval. All other analyses were conducted regarding data from unrelated treatments versus control. All the data were assumed to have a normal distribution, and were analysed initially using a basic analysis of variance (one way ANOVA). Any significant difference was established using a 95% confidence interval ($P<0.05$).

Results and Discussion

1. Decolorisation experiment: A preliminary study

The general response of lettuce seedlings to MB stress was reduction in % germination by about 20%. The radicle length was also decreased under MB stress as compared to de-ionised water as shown in Figures 7 and 8.

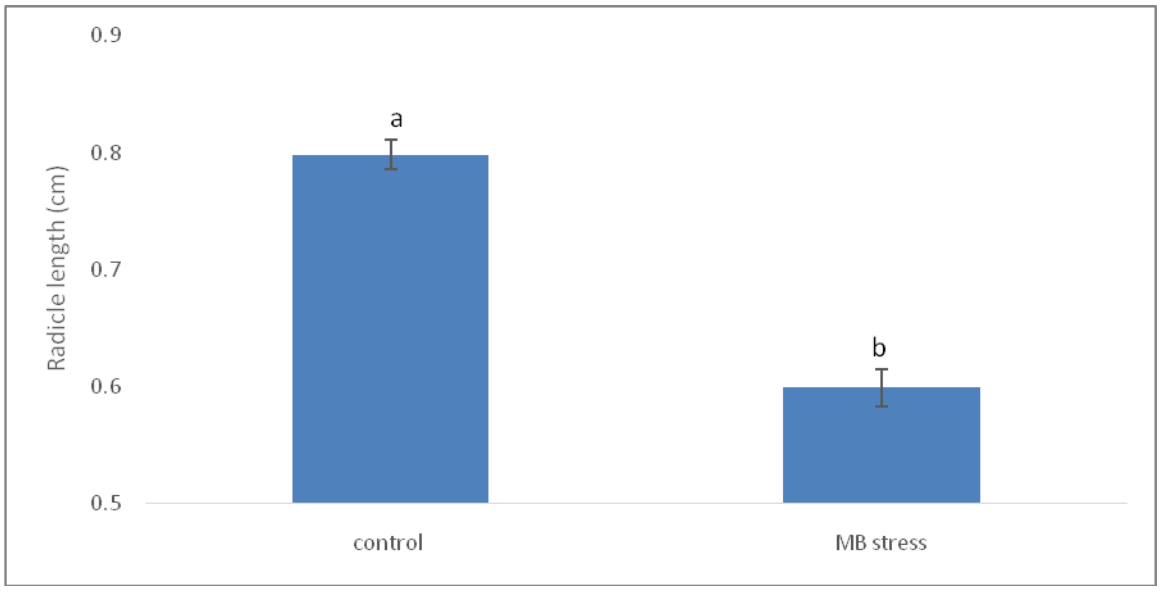

Figure 7: Change in radicle length of lettuce seedlings in response to MB (40 mg/L) stress in comparison to de-ionised water after 48 hours of stress. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

Figure 8: Lettuce seedlings after 48 hours of dye stress.

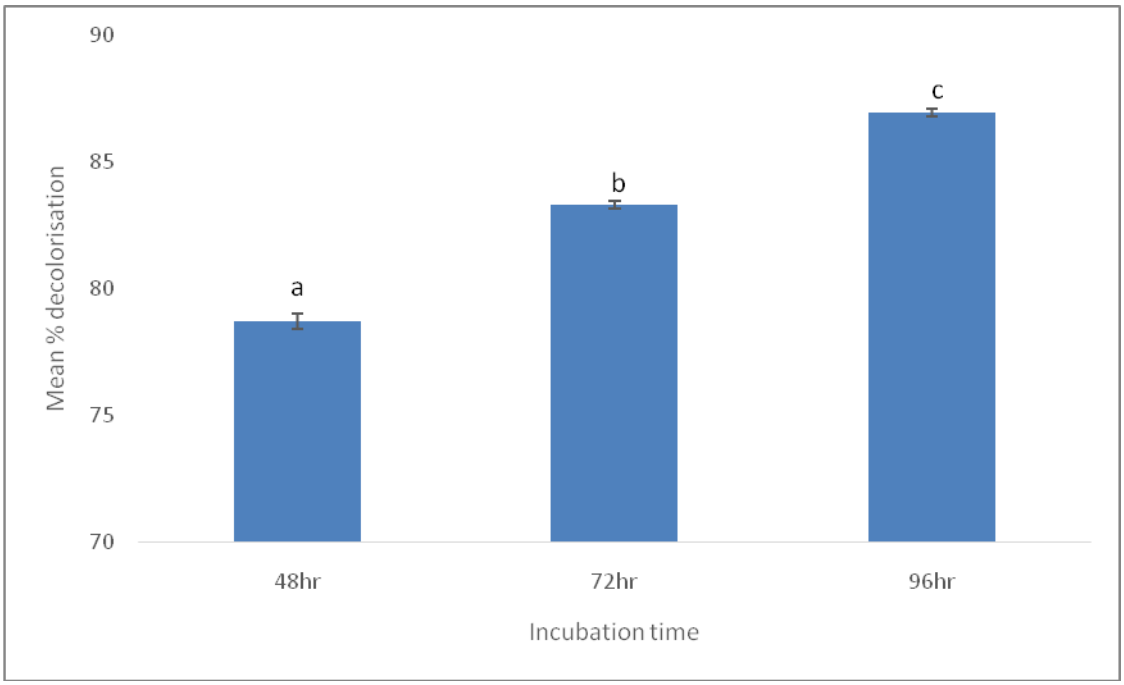

Figure 9: Decolorisation capacity of Lettuce seedlings. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

Lettuce seedlings (mainly the radicles) were able to decolorise 40 mg/L MB solution by 78% in the first 48 hours and up to 86% after 96 hours (Figure 9). After carrying out the preliminary decolorisation experiments with lettuce seeds, it was clear that lettuce had the ability to decolorise MB efficiently. The reduction in radicle length confirms the toxicity of MB. As reported earlier by Patil et al. (2009), due to the toxic effect of different dyes on plants, the plant's metabolic activities and growth rates could also be affected.

1.1 Direct treatment experiments

1.1.1 Screening experiment

All the three types of seeds (lettuce, ryegrass and wheat) demonstrated different decolorisation capacity under MB (40 mg/L) stress. Lettuce showed maximum decolorisation activity while ryegrass showed the minimum (Figure 10).

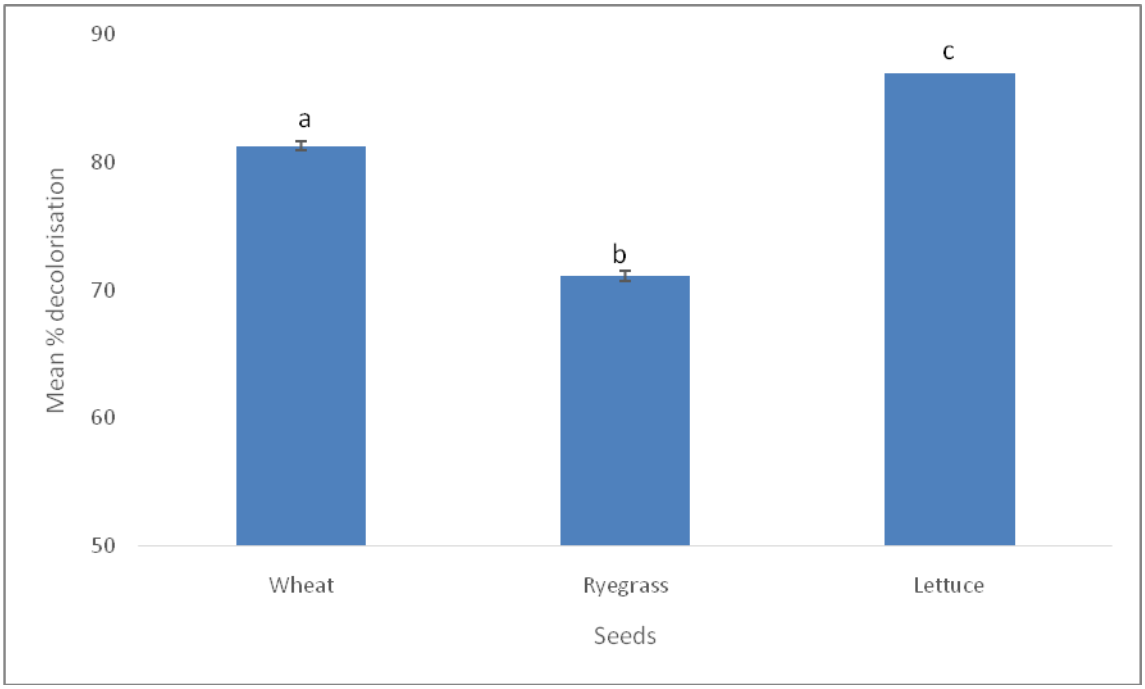

Figure 10: Decolorisation capacity of various seeds under MB (40mg/L). Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

After incubation in 40 mg/L MB for 48 g there was no change in % germination for wheat seeds while lettuce and ryegrass both showed decreased % germination. The % germination in lettuce was around 70% while in ryegrass was about 50%. All the three seeds showed decrease in radicle lengths when compared with controls by more than 40% (figure 11). Similar behavior wherein different plants exhibited different capabilities of phytoremediation is explained by Mbuligwe (2005).

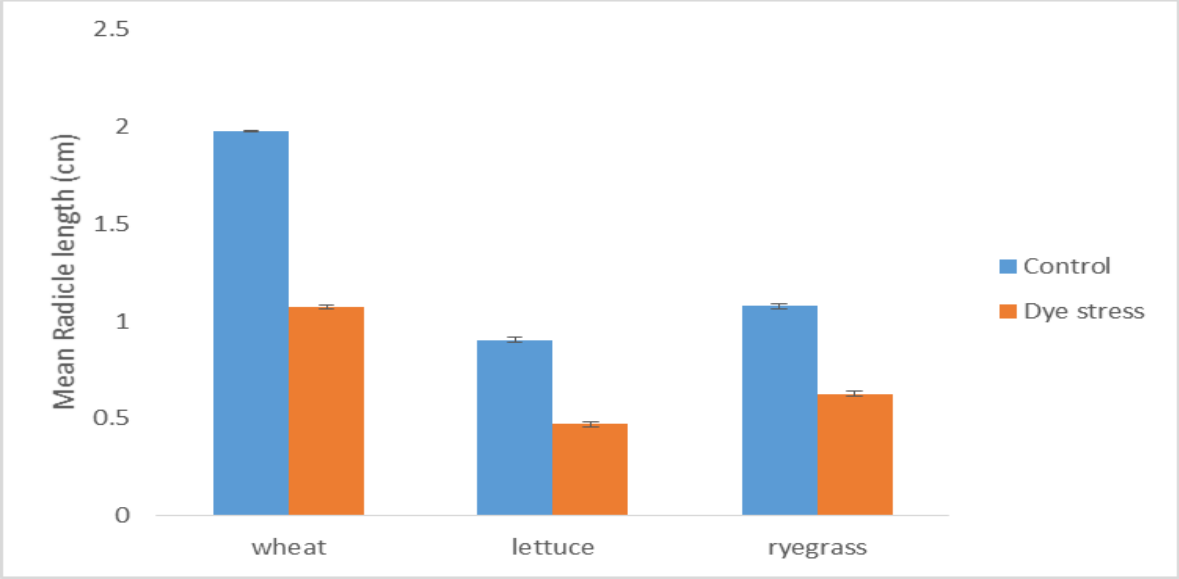

Figure 11: Mean radicle lengths of wheat , lettuce and ryegrass seedlings grown in 40 mg/L MB dye stress and de-ionised water control. Error bars signify standard error of the mean.

1.1.2 Effect of increasing dye concentrations

Lettuce seeds decolorised increasing concentrations of MB with efficiency varying from 28 to 80% (Figure 12). The decolorisation potential was inversely proportional to the increase in MB concentration with 80.99% decolorisation of 40 mg/L in 48 hours, whereas 400 mg/L showed only 28.73% decolorisation in the same time period. A matching trend was reported by Patil et al. (2009) and Jha et al. 2015 for Reactive red 198 decolorisation using hairy root cultures of *Tagetes patula* and for Reactive black 8 decolorisation using hairy roots of *Physalis minima L.*, respectively.

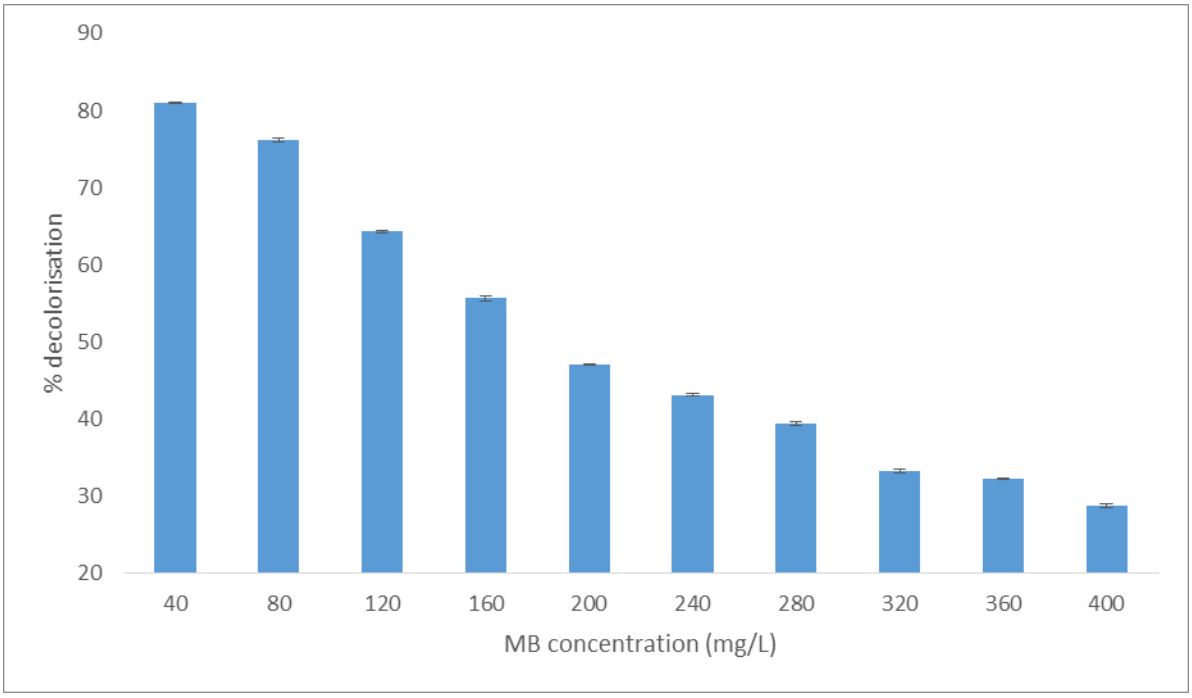

Figure 12: Effect of increasing MB concentrations on the % decolorisation by lettuce seedlings after 48 hours of stress. Error bars signify standard error of the mean.

The increase in dye concentration also affected the % germination and radicle length of Lettuce seeds. A significant decrease in radicle length (approximately 50%) was observed in 400 mg/L MB when compared to de-ionised water control.

1.1.3 Increased number of lettuce seeds

Increase in the number of lettuce seeds did not impact the % germination or radicle length significantly but the % decolorisation was increased to approximately 88% within the first 48 hours. This is about an 8% increase when compared to decolorisation using 10 seeds for same concentration of dye and same time period. Thus by using more seeds efficient decolorisation may be enhanced. Similar results were reported by Kabra et al. (2011).

1.1.4 Effect of repetitive stress

The change in the capacity of the same lettuce seedlings to decolorise MB under repetitive cycles of stress exposure was observed. A significant decrease in % decolorisation was observed after

the third stress cycle. The % decolorisation after first stress cycle was around 80% while after repetitive stress for 3 cycles of 48 hour each the % decolorisation was reduced to about 43% (Figure 13).

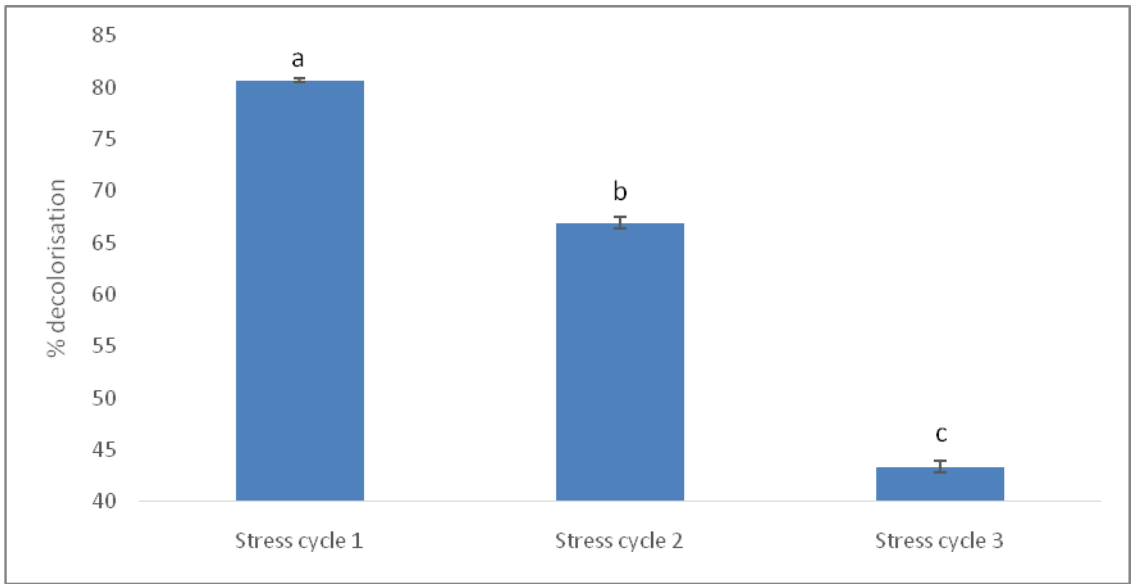

Figure 13: Effect of repetitive stress on decolorisation capacity of lettuce seeds. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

The discharge of dyeing waste is a continuous process in textile industries, thus it is important that the plant would have a capacity to degrade this continuous influx of dye. Therefore the consequence of repetitive dye stress investigated. In dye phytoremediation studies by Movafeghi et al. (2013), Khataee et al. (2013) and Torbati et al. (2015), the plants showed no decrease in the dye decolorisation efficiency under continuous stress. But in the present study the efficiency of lettuce seedlings was found to decrease with continuous stress exposure. This can be due to the toxic effect of dyes on the plant growth which in turn may be affecting its decolorisation capacity.

1.1.5 Decolorisation under aseptic conditions

There was no significant difference observed in experiments performed in aseptic conditions and experiments performed in non-sterile conditions with respect to the % decolorisation, %

germination and radicle length of lettuce seeds. The % germination was found to be about 70% and the reduction in radicle length was about 25%. The decolorisation capacity was recorded to be 76.5% after 48 hours of stress. This indicates that the decolorisation of dye is solely due to the plant and not other micro-organisms that might be associated with the lettuce root surface.

1.2 Pre-treatment experiments

1.2.1 Effect of pre-germination on increasing dye concentration

Unlike the previously described experiments, lettuce seeds were not sown and germinated in 40 mg/L MB. But instead, seeds were first sown and germinated in deionized water (called pre-germination treatment) before the seedlings were incubated in MB. With increasing MB concentrations, a significant decrease in the decolorisation capacity of lettuce seedlings was observed (Figure 14).

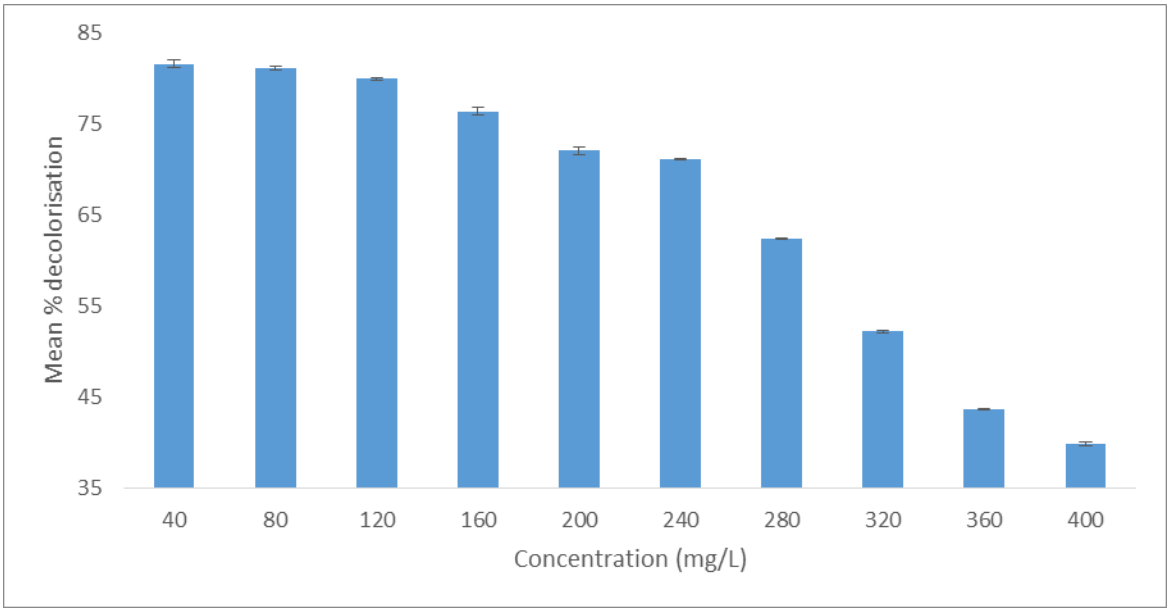

Figure 14: Effect of increasing MB concentrations on the % decolorisation by pre-germinated lettuce seedling after 48 hours of stress. Error bars signify standard error of the mean.

The increase in dye concentration also affected radicle length of lettuce seeds. A significant decrease (approximately 48%) was observed in the radicle length in of lettuce seeds in 400mg/L MB when compared to de-ionised water control.

With an increase in the concentration of MB, a decrease was observed in the decolorisation potential of lettuce. Similar results have been reported in the case of *G. pulchella* for dye Green HE4B dye (Kabra et al., 2011) and *B. malcolmii* for the dye Direct Red 5B (Kagalkar et al. 2009). A higher dye concentration might be toxic to the plant, resulting in an impact on the growth rate and enzymatic system, in turn leading to reduction of the decolorisation potential of the plant (Kabra et al., 2011; Patil et al., 2009).

Also in the present study, it was found that pre-germinating the seeds had a positive effect on their decolorisation capacity at higher MB concentrations. Incubation of directly sown lettuce seeds with 400 mg/L MB only 28.73% decolorisation of MB was observed after 48 hours, while in the pre-germination experiment after the lettuce seedlings were incubated for 48 hours with 400 mg/L MB the decolorisation % was 39.89%. The reason behind this is still unknown.

1.2.2 Effect of seed coat

The presence or absence of seed coat of lettuce seeds did not affect the % germination or radicle length, but the % decolorisation was increased by around 5% in absence of seed coat (Figure 15). This is contrary to the results expected as ideally the seed coat should have been adsorbing a certain amount of dye and thus the decolorisation was expected to be higher in the seed with seed coat. It seems that seed coat-mediated physical adsorption of MB played a minor role in the decolorisation of MB by lettuce seeds / seedlings. This has not been reported previously.

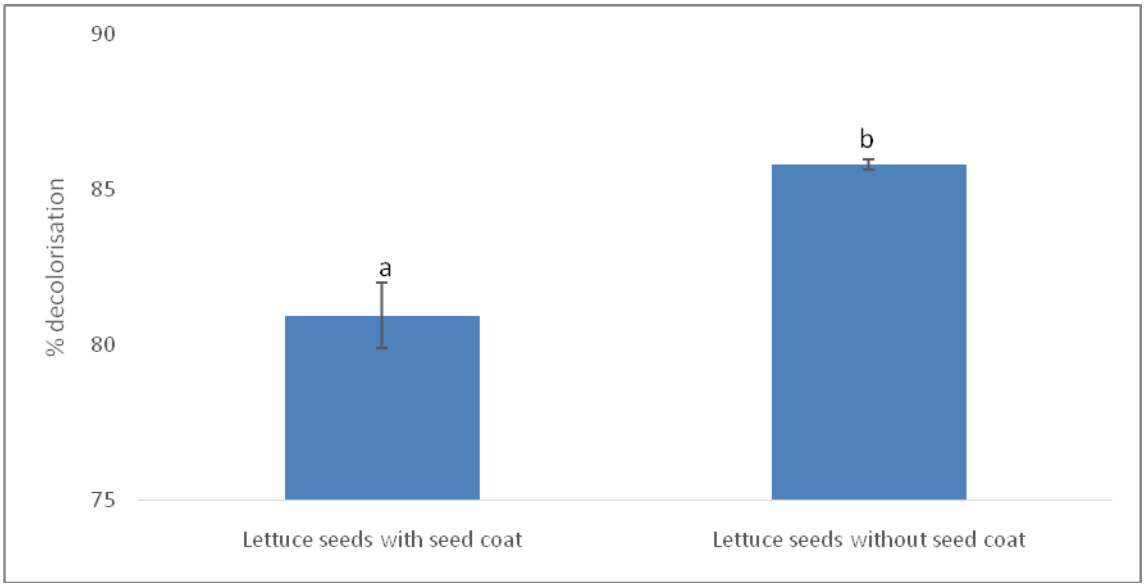

Figure 15: Effect of Seed coat on the % decolorisation of Lettuce after 48hours of stress. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

1.2.3 Effect of MB dissolved in spent water

The radicle length of lettuce seeds grown in MB prepared using spent water (in which lettuce seeds were germinated for 48 h) was found to increase marginally when compared to the radicle length of lettuce seeds grown in MB prepared using fresh de-ionised water. The % decolorisation by lettuce seeds grown in MB prepared using spent water was higher by around 2.6% as compared to % decolorisation by lettuce seeds grown in MB prepared using fresh de-ionised water (Figure 16). From these results it seems that the lettuce seeds might have released certain biomolecules which could have aided MB decolorisation although the concentration of these molecules was probably too diluted for a more substantial effect to be detected.

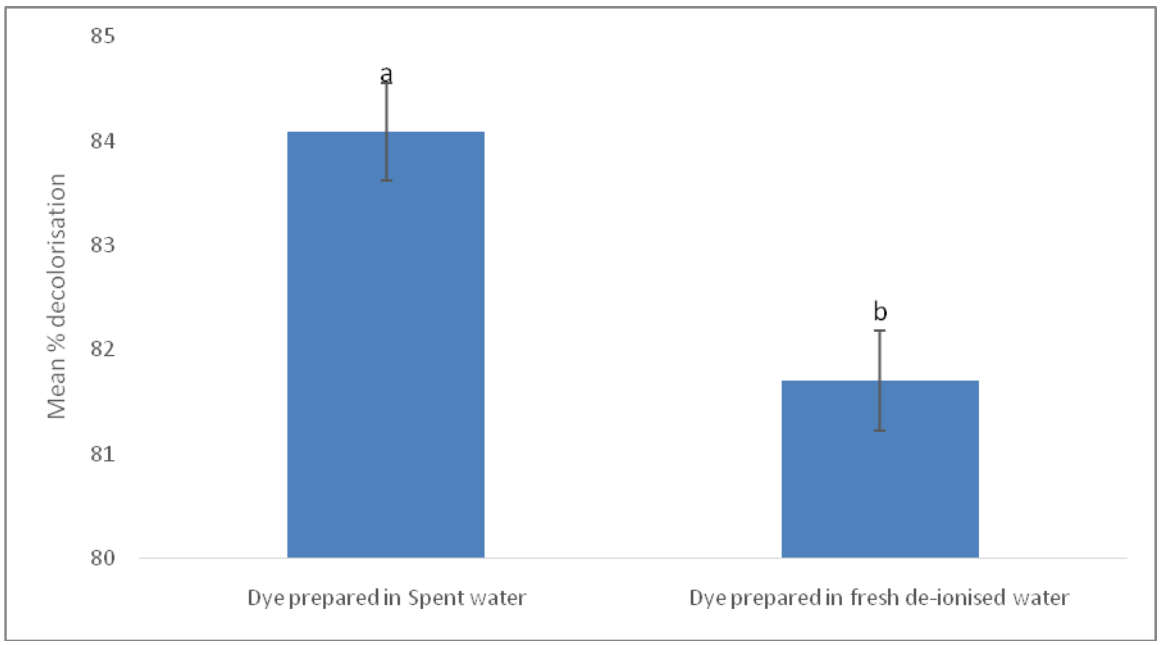

Figure 16: Effect of using fresh and spent water (in MB preparation) on the % decolorisation of Lettuce after 48hours of stress. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

1.2.4 Increased number of lettuce seeds

Using more lettuce seedlings in the pre-germination treatment did not affect the % germination or radicle length significantly but the % MB decolorisation was increased to approximately 88.5% within the first 48 hours under stress using 20 lettuce seeds as compared to 80% using 10 seeds. Thus it can be said that by using increased number of seeds an efficient decolorisation could be achieved in a shorter time period. Similar results were reported by Kabra et al. (2011) in the case of *G. pulchella* for decolorisation of the dye Green HE4B dye.

1.3 Treatments under controlled pH conditions

The experiments were repeated in MES buffer (pH 6.0) for comparison with the results from the previous experiments in which de-ionised water was used to prepare the dye solution or as control. No significant impact on seedling growth, % germination or decolorisation capacity was observed in controlled pH conditions or otherwise. The results of the direct and pre-treatment experiments using MB prepared in MES buffer (pH 6.0) confirmed that the observed decolorisation in the present study were not related to variation in pH.

2. Experiment using Kowhai seeds

The general response of Kowhai seedlings to MB stress was reduction in the radicle length as compared to de-ionised water as shown in Figures 17 & 18.

Figure 17: Kowhai seeds after 48 hours of dye stress.

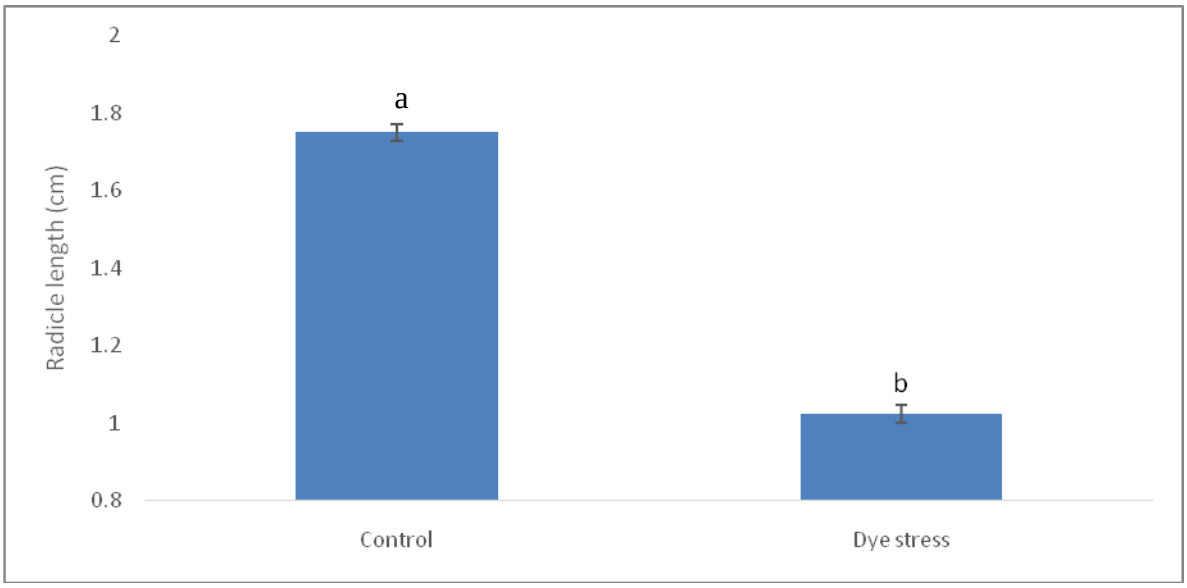

Figure 18: Change in radicle length of Kowhai seedlings due to MB (40mg/L) stress in comparison to de-ionised water after 48hours of stress. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

After carrying out the preliminary decolorisation experiments with Kowhai seeds, it was clear that Kowhai seedlings also had the ability to decolorise MB efficiently. Kowhai seedlings (mainly the radicles) were able to decolorise 40 mg/L MB solution by 77.5% in the first 48 hours. The reduction in Kowhai radicle length is related the toxic nature of MB. As reported earlier by Patil et al. (2009), due to the toxic effect of dyes on plants, the plant's metabolic activities and growth rates are also affected. Also as the seeds were surface sterilised and the experiment was carried out under aseptic conditions. Thus MB decolorisation by Kowhai seedlings is not due to other microorganisms that might be associated with plant root surface.

3. Enzyme assays

The major mechanism of biodegradation in living cells is attributed to lignin-modifying enzymes like laccase, manganese peroxidase, lignin peroxidase, etc (Raghukumar et al. 1996). Different enzymes in plant and microbial sources have been found to be involved in the degradation of dyes (Kabra et al., 2011). The induction of extracellular and intracellular enzymes was correlated with their involvement in the process of dye degradation. Patil et al. (2009) highlighted the combined effect of oxidative and reductive enzyme during dye degradation. Induction in the intracellular activity of laccase enzyme was reported in *B. juncea* on exposure to the dye Reactive Red 2 by Ghodake et al. (2009). The role of peroxidases has been recorded in the plant *Phragmites australis* used for the degradation of the dye Acid Orange 7 by Carias et al. (2007). The induction of the enzymes peroxidase, tyrosinase, and 2,6-dicholorophenol-indophenol (DCIP) reductase has been reported during the decolorisation of the dye Direct Red 5B in *B. malcolmii* (Kagalkar et al. 2009). The activities of the enzymes lignin peroxidase, laccase, tyrosinase, and DCIP reductase were induced on exposure to the dye Green HE4B. This indicates that these enzymes might be induced in response to the presence of the dye Green HE4B and could be important for the degradation of the dye (Kabra et al., 2011). The specific enzyme activities of laccase and lignin peroxidase of lettuce seedlings studied in the present study were determined.

3.1 Laccase assay

Very small quantities of laccase enzyme activity were found in the intracellular extracts for both lettuce seedlings under control and dye stress conditions. The extracellular extracts had a higher

level of laccase activity under both conditions. The laccase activity was found to be higher under dye stress as compared to the control in both extracellular and intracellular extracts. There was approximately a tenfold increase in the extracellular enzyme activity as compared to intracellular enzyme activity under dye stress but there was only a five-fold increase in control (Figure 19).

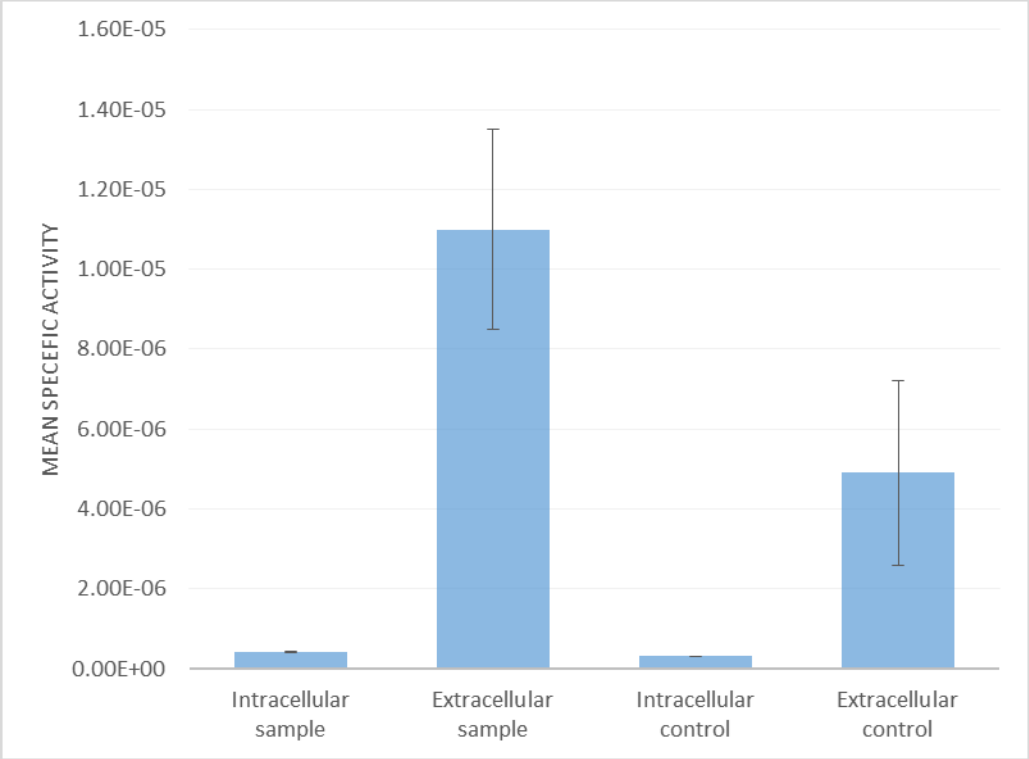

Figure 19: Specific enzyme activity for laccase in extracellular and intracellular extracts under dye stress and control conditions for 48 hours. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$). The mean specific activity is expressed in µmol min^{-1} mg^{-1}.

3.2 Lignin peroxidase assay

Very small quantities of lignin peroxidase enzyme activity was found in the intracellular extracts for both Control and dye stress samples. The extracellular extracts had higher quantity of Laccase enzyme activity for the dye stress sample while no extracellular Lignin Peroxidase activity was recorded in the control samples. The Lignin Peroxidase enzyme activity was found to be higher in the dye stress samples as compared to the control samples in both extracellular

and intracellular extracts. There was approximately a tenfold increase in the extracellular enzyme activity as compared to intracellular enzyme activity in the dye stress samples (Figure 20).

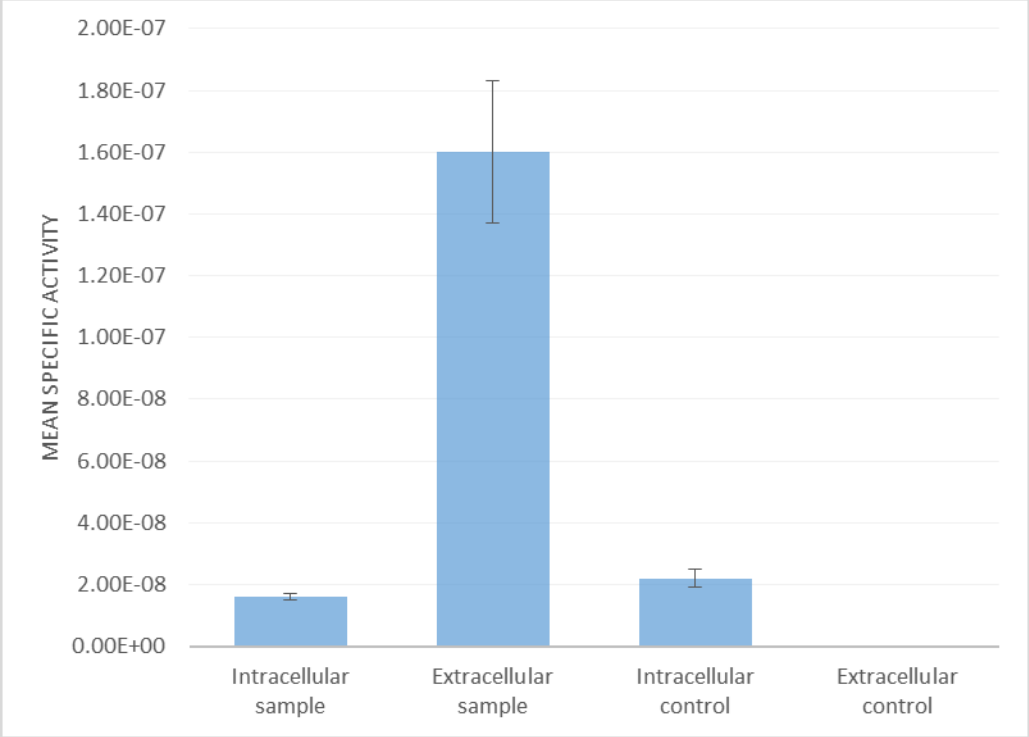

Figure 20: Specific enzyme activity for lignin peroxidase in extracellular and intracellular extracts for dye stress and control samples after 48hrs. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$). The mean specific activity is expressed in µmol min^{-1} mg^{-1}.

4. Cyclic voltammetry

Oxidised methylene blue is blue in color but the reduced form is colorless. Thus when decolorisation of MB was observed, it might just be present in its reduced form. To investigate this possibility cyclic voltammetry was performed. Cyclic voltammetry analysis showed that the spectrum of 40 mg/L MB before incubation with lettuce seedlings gave two peaks showing the fully oxidized and fully reduced states. Analysis of the test solutions obtained after full decolorisation of MB by 35 lettuce seedlings showed that both these peaks had been removed (Figure 21). The absence of the peaks in the voltammogram of decolorised solution thus

confirms the decolorisation and removal of MB from the solution in the presence of lettuce seedlings.

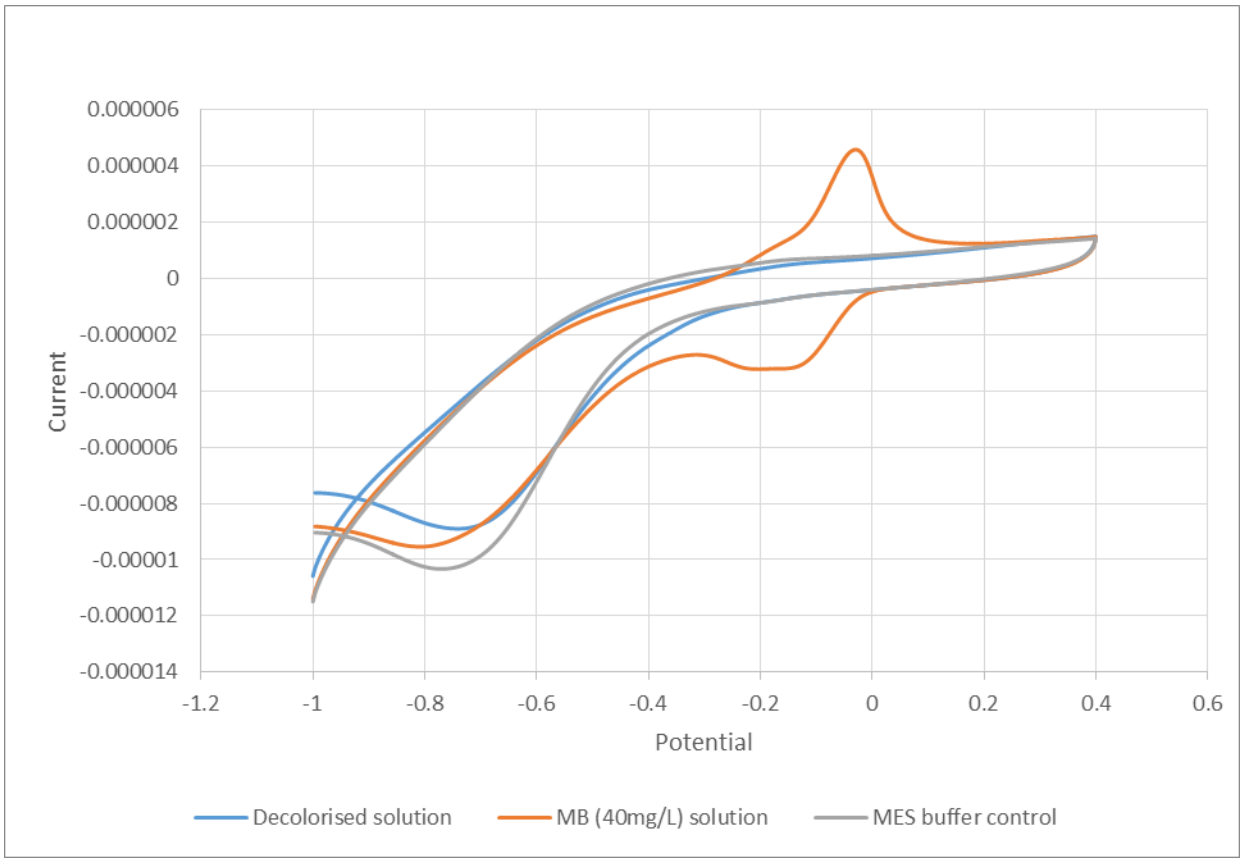

Figure 21: Voltammograms of MB solution, decolorised solution and MES buffer.

5. UV-Visible Spectroscopy

UV visible spectral analysis showed that the absorption spectrum of the 40 mg/L MB before incubation with lettuce seedlings showed three peaks: One in the visible region at 585 nm and another two in the UV region at 290 and 245 nm respectively. UV visible spectral analysis of the test solutions obtained after full decolorisation of MB by 35 lettuce seedlings showed the peaks in the visible region had been removed, while the one near UV region (about 245 nm) showed an increase in absorbance (Figure 22). This confirms decolorisation by the plant. Also the increase in the absorbance in UV range and the disappearance of the maximum absorbance peak indicated that the color removal by lettuce may attribute towards biodegradation rather than simple

decolorisation of MB. Similar results were reported by Jha et al. (2015); Saratale et al. (2009) and Patil et al. (2012).

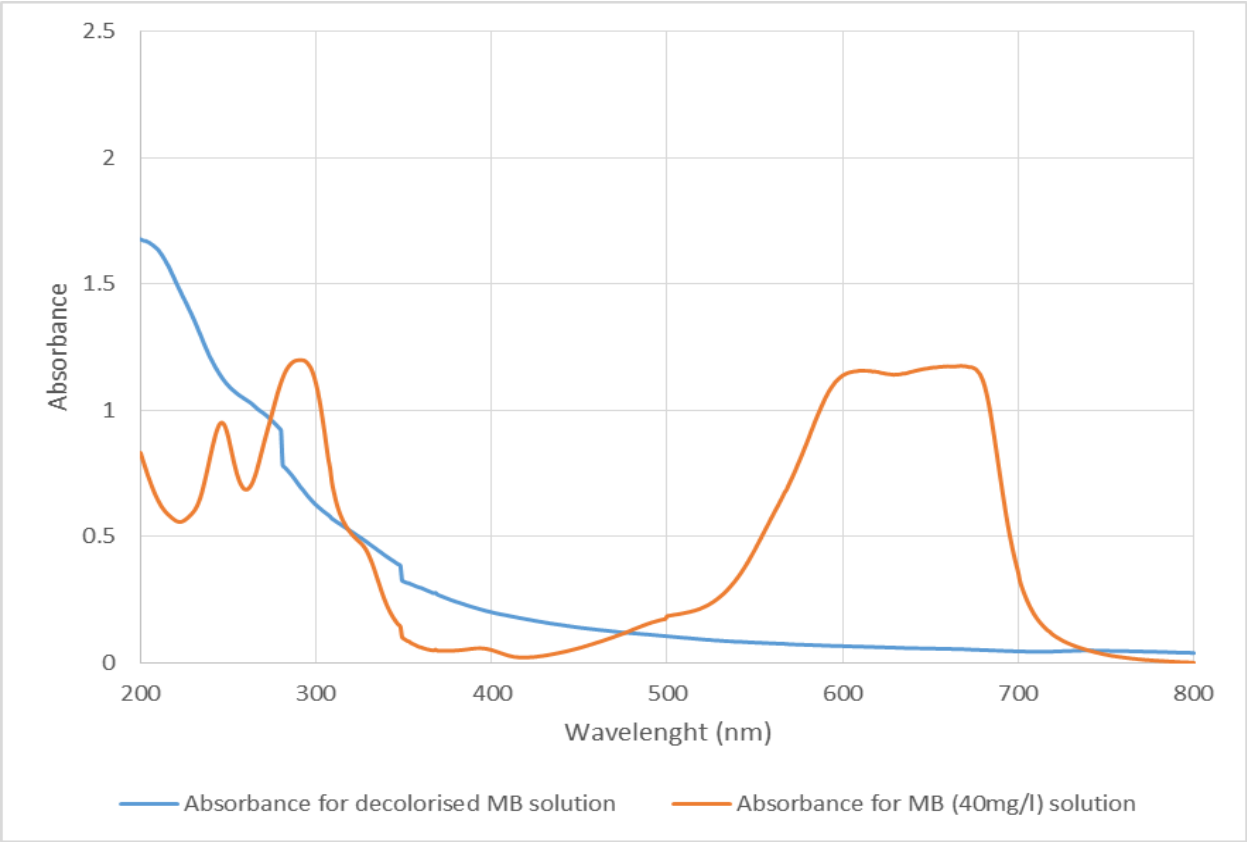

Figure 22: UV Visible spectral scans of MB solution (before lettuce seedling incubation) and decolorised solution following incubation with lettuce seeds.

6. Phytotoxicity studies

Lower % germination (65%) was observed in ryegrass seed under MB stress while the % germination was increased to 90% in the decolorised MB solution. The fresh and spent de-ionised water (previously used for germination of 35 lettuce seeds) also showed 90% germination. The 40 mg/L untreated MB solution inhibited the radicle growth of ryegrass seeds when compared to the fully decolorised solution obtained following incubation with 35 lettuce seeds, spent water and fresh de-ionised water. The mean radicle length of seedlings grown in MB solution was reduced by 70% as compared to ryegrass seedlings growing in spent de-ionised water. Whereas the mean radicle length of ryegrass seedling growing in decolorised dye solution

was reduced by 18% as compared to ryegrass seedlings growing in spent de-ionised water. The mean radicle length of seedlings growing in spent de-ionised water was approximately 40% longer than the seedlings growing in fresh de-ionised water (Figure 23).

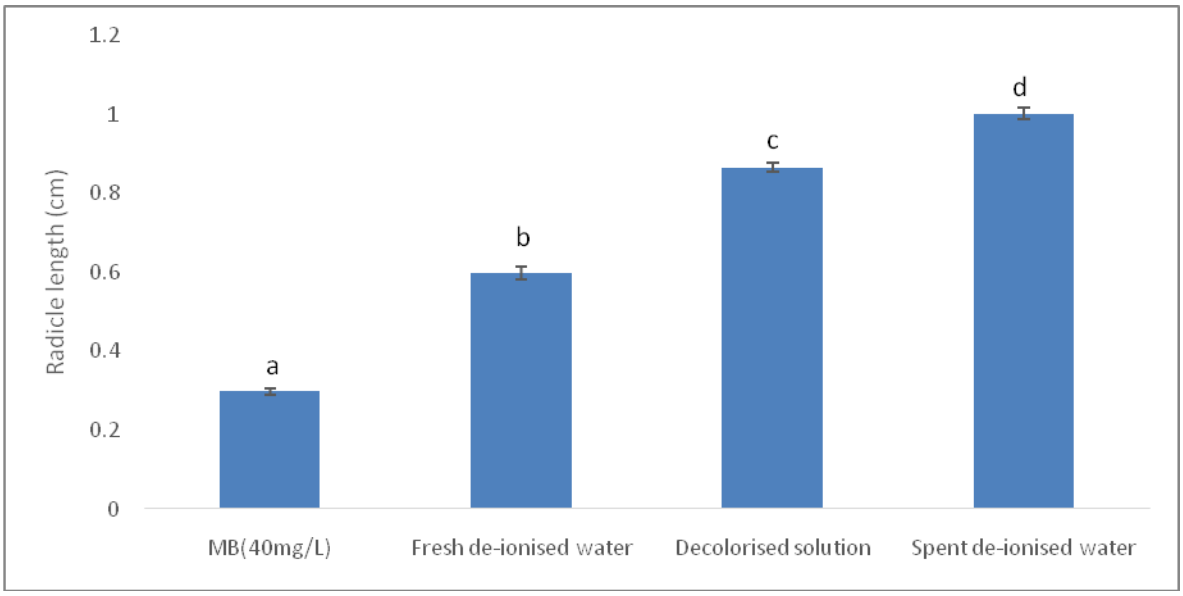

Figure 23: Phytotoxicity studies using ryegrass seeds. Error bars signify standard error of the mean. Statistically significant results are designated different letters ($p<0.05$).

The length of the ryegrass seedling radicle was reduced when grown under dye stress as compared to those grown in de-ionised water or decolorised MB solution. This indicates the toxic nature of MB and the metabolites formed after decolorisation of MB did seem to be substantially less phytotoxic. Similar results were seen when lettuce seedlings were grown in fully decolorised MB solution. Also this experiment replicated the results of the previous experiment (figure 16) suggesting that the presence of some biomolecule produced by lettuce seedlings that could aid the growth and decolorisation capacity of lettuce.

Conclusion

Over the last years due to stringent legislations aiming environmental protection, several systems of dye removal have been developed and explored mostly using microorganisms. The use of plant systems to remediate these dyes has not been ventured into to that great extent. The use of lettuce and kowhai to achieve this objective has not been a subject of investigation till yet.

The main aim of this study was to investigate the potential of lettuce (*Lactuca sativa* L., variety 'Great Lakes') and Kowhai (*Sophora* spp.) to decolorize Methylene blue dye. The present study led to the concluson that both plants have potential to degrade methylene blue effectively at 40 mg/L concentration in 48 hours. The dye had toxic effect on the seedlings as apparent with the decrease in radicle length of seedlings after germination. Lettuce was also reported decolorize higher concentrations of the dye to a certain extent. Thus these plants could be exploited for their phytoremediation capabilities with following major advantages:

1. Low cost of the decolorisation system
2. Wide availability of plant
3. Easy growth conditions

Study also showed that the increase in the number of seeds increased the decolorisation. When pre-germinated, the seeds had increased decolorisation capacity. Further analysis of the decolorised solution using UV Visible spectroscopy and cyclic voltammetry confirm the absence of the dye from the solution rather than just being reduced into a colorless form. The induction of laccase and lignin peroxidase enzymes during dye decolorisation was also seen, indicating towards an active enzyme system under dye stress. Given the outcomes of the present study, lettuce and kowhai have potential to decolorise MB.

Future directions

This work provides a starting point for more intensive study to better understand the use of lettuce and kowhai in phytoremediation of other textile dyes, as well as future clues for the biotechnological optimization of the method. However, further in-depth investigations are required to elucidate the mechanism of MB degradation by the plant. Also the potential of both plants to decolorise other textile dyes would be an interesting aspect for further research. Techniques like Fourier transform infrared spectroscopy (FTIR), Gas Chromatography Mass Spectrometry (GCMS) and High Performance Liquid Chromatography (HPLC) may shed light on the metabolites formed after the dye decolorisation. It would be particularly exciting to investigate the genes involved in decolorisation, which opens up the possibility of genetic manipulation in order to increase the system performance. Another phase to be investigated is the decolorisation potential of these plants in consortium with other plants and micro-organisms. The study of effects of several other parameters like presence of mixture of textile dyes, other textile additives etc, would reveal more aspects of this system. This would also allow the development of a pilot treatment plant to apply in a textile industry.

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